

# PATENT COOPERATION TREATY

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: YURIY P. STERCHO  
SMITHKLINE BEECHAM CORPORATION  
CORPORATE INTELLECTUAL PROPERTY, UW2220  
709 SWEDELAND ROAD, P.O. BOX 1539  
KING OF PRUSSIA, PA 19406-0939

## PCT

### NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing (day/month/year)		26 OCT 2000
Applicant's or agent's file reference P50800		IMPORTANT NOTIFICATION
International application No. PCT/US99/15366	International filing date (day/month/year) 07 JULY 1999	Priority Date (day/month/year) 07 JULY 1998
Applicant SMITHKLINE BEECHAM CORPORATION		

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

BRIAN J. DAVIS

Telephone No. (703) 308-2351

# PATENT COOPERATION TREATY

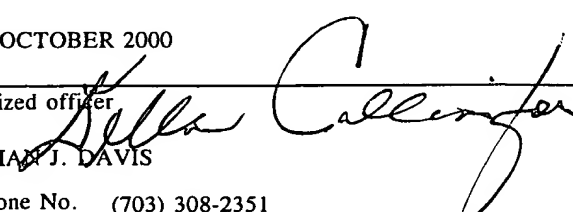
## PCT

### INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P50800	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/15366	International filing date ( <i>day/month/year</i> ) 07 JULY 1999	Priority date ( <i>day/month/year</i> ) 07 JULY 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant SMITHKLINE BEECHAM CORPORATION		

1.	This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2.	This REPORT consists of a total of <u>4</u> sheets.  <input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).  These annexes consist of a total of <u>0</u> sheets.
3.	This report contains indications relating to the following items:  I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input type="checkbox"/> Non-establishment of report with regard to novelty, inventive step or industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input type="checkbox"/> Certain observations on the international application

Date of submission of the demand  28 JANUARY 2000	Date of completion of this report  03 OCTOBER 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer  BRIAN J. DAVIS
Facsimile No. (703) 305-3230	Telephone No. (703) 308-2351

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/15366

**I. Basis of the report**

## 1. With regard to the elements of the international application:\*

- ☒ the international application as originally filed
- ☒ the description:  
pages 1-12 , as originally filed  
pages NONE , filed with the demand  
pages NONE , filed with the letter of \_\_\_\_\_
- ☒ the claims:  
pages 13-16 , as originally filed  
pages NONE , as amended (together with any statement) under Article 19  
pages NONE , filed with the demand  
pages NONE , filed with the letter of \_\_\_\_\_
- ☒ the drawings:  
pages NONE , as originally filed  
pages NONE , filed with the demand  
pages NONE , filed with the letter of \_\_\_\_\_
- ☒ the sequence listing part of the description:  
pages NONE , as originally filed  
pages NONE , filed with the demand  
pages NONE , filed with the letter of \_\_\_\_\_

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language \_\_\_\_\_ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in printed form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

- ☒ the description, pages none
- ☒ the claims, Nos. none
- ☒ the drawings, sheets/fig none

5. ☒ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).\*\*

\* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

\*\*Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement****1. statement**

Novelty (N)	Claims	<u>1-10</u>	YES
	Claims	<u>none</u>	NO
Inventive Step (IS)	Claims	<u>2, 3, 9, 10</u>	YES
	Claims	<u>1,4-8</u>	NO
Industrial Applicability (IA)	Claims	<u>1-10</u>	YES
	Claims	<u>none</u>	NO

**2. citations and explanations (Rule 70.7)**

Claims 1 and 4-8 lack an inventive step under PCT Article 33(3) as being obvious over Li. Li teaches amine-reactive forms of a luminescent diethylenetriaminepentaacetic acid chelate of terbium and europium, attachment to DNA and energy transfer measurements. That is, Li teaches the the instant compounds for the instantly claimed utility.

Claims 2, 3, 9 and 10 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the instant R1 moieties of the compounds nor the instant kit.

Claims 1-10 have industrial applicability in the manufacture of new bioaffinity assays.

----- NEW CITATIONS -----  
NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/15366

**Supplemental Box**

(To be used when the space in any of the preceding boxes is not sufficient)

Sheet 10

Continuation of: Boxes I - VIII

**CLASSIFICATION:**

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(7): CO7C 229/42, 229/76; CO7D 219/04, 311/88, 491/052 and US Cl.: 546/89, 103; 549/225, 226; 560/39, 41; 562/450

**I. BASIS OF REPORT:**

5. (Some) amendments are considered to go beyond the disclosure as filed:  
NONE

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PCT  
REQUEST

The undersigned requests that the present  
international application be processed  
according to the Patent Cooperation Treaty

For receiving Office use only

<b>PCT/US 99 15366</b>	
International Application No:	
(07.07.99)	07 JUL 1999
International Filing Date	
<b>PCT INTERNATIONAL APPLICATION RO/US</b>	
Name of receiving Office and "PCT International Application"	

Applicant's or agent's file reference  
**P50800**

<b>Box No. I TITLE OF INVENTION</b>			
NOVEL FLUORESCENT LANTHANIDE CHELATES			
<b>Box No. II APPLICANT</b>			
<b>Name and Address:</b> (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)		<input type="checkbox"/> This person is also inventor	
SmithKline Beecham Corporation One Franklin Plaza Philadelphia, Pennsylvania 19103 United States of America		Telephone No. 610-270-5018 Facsimile No. 610-270-5090 Teleprinter No.	
State (i.e. country) of nationality: United States of America		State (i.e. country) of residence: United States of America	
This person is applicant for the purposes of <input type="checkbox"/>		all designated States <input checked="" type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental box <input type="checkbox"/>	
<b>Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS</b>			
<b>Name and Address:</b> (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)		<input type="checkbox"/> This person applicant only	
Chan, George Wai-Kin 249 Wiltshire Road Wynnewood, Pennsylvania 19096 United States of America		<input checked="" type="checkbox"/> applicant and inventor	
		<input type="checkbox"/> inventor only (if this check-box is marked, do not fill in below)	
State (i.e. country) of nationality: United States of America		State (i.e. country) of residence: United States of America	
This person is applicant for the purposes of <input type="checkbox"/>		all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental box <input type="checkbox"/>	
<b>Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE</b>			
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:		<input checked="" type="checkbox"/> agent <input type="checkbox"/> common representative	
<b>Name and Address:</b> (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)		Telephone No.	
Stercho, Yuriy P. SMITHKLINE BEECHAM CORPORATION Corporate Intellectual Property, UW2220 709 Swedeland Road, P.O. Box 1539 King of Prussia, Pennsylvania 19406-0939 United States of America		610-270-5018	
		Facsimile No.	
		610-270-5090	
		Teleprinter No.	
<input type="checkbox"/> Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent			
<input checked="" type="checkbox"/> Further applicants and/or (further) inventors are indicated on a continuation sheet.			

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS			
<i>If none of the following sub-boxes is used, this sheet is not to be included in the request</i>			
Name and Address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)  <b>Hertzberg, Robert P.</b> <b>121 Longfields Way</b> <b>Downington, Pennsylvania 19335</b> <b>United States of America</b>		<input type="checkbox"/> This person applicant only  <input checked="" type="checkbox"/> applicant and inventor  <input type="checkbox"/> inventor only (if this check-box is marked, do not fill in below)	
State (i.e. country) of nationality: <b>United States of America</b>		State (i.e. country) of residence: <b>United States of America</b>	
This person is applicant for the purposes of <input type="checkbox"/> all designated States		<input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental box	
Name and Address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)  		<input type="checkbox"/> This person applicant only  <input type="checkbox"/> applicant and inventor  <input type="checkbox"/> inventor only (if this check-box is marked, do not fill in below)	
State (i.e. country) of nationality:		State (i.e. country) of residence:	
This person is applicant for the purposes of <input type="checkbox"/> all designated States		<input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental box	
Name and Address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)  		<input type="checkbox"/> This person applicant only  <input type="checkbox"/> applicant and inventor  <input type="checkbox"/> inventor only (if this check-box is marked, do not fill in below)	
State (i.e. country) of nationality:		State (i.e. country) of residence:	
This person is applicant for the purposes of <input type="checkbox"/> all designated States		<input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental box	
Name and Address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)  		<input type="checkbox"/> This person applicant only  <input type="checkbox"/> applicant and inventor  <input type="checkbox"/> inventor only (if this check-box is marked, do not fill in below)	
State (i.e. country) of nationality:		State (i.e. country) of residence:	
This person is applicant for the purposes of <input type="checkbox"/> all designated States		<input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental box	
Name and Address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)  		<input type="checkbox"/> This person applicant only  <input type="checkbox"/> applicant and inventor  <input type="checkbox"/> inventor only (if this check-box is marked, do not fill in below)	
State (i.e. country) of nationality:		State (i.e. country) of residence:	
This person is applicant for the purposes of <input type="checkbox"/> all designated States		<input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental box	

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet

## Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

## Regional Patent

- ☐ AP ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland; UG Uganda, ZW Zimbabwe and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☐ EA Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☐ OA OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line).....

## National Patent (if other kind of protection or treatment desired, specify on dotted line):

- |   |   |
|---|---|
| <input type="checkbox"/> AL Albania                               | <input type="checkbox"/> LS Lesotho                                   |
| <input type="checkbox"/> AM Armenia                               | <input type="checkbox"/> LT Lithuania                                 |
| <input type="checkbox"/> AT Austria                               | <input type="checkbox"/> LU Luxembourg                                |
| <input type="checkbox"/> AU Australia                             | <input type="checkbox"/> LV Latvia                                    |
| <input type="checkbox"/> AZ Azerbaijan                            | <input type="checkbox"/> MD Republic of Moldova                       |
| <input type="checkbox"/> BA Bosnia and Herzegovina                | <input type="checkbox"/> MG Madagascar                                |
| <input type="checkbox"/> BB Barbados                              | <input type="checkbox"/> MK The former Yugoslav Republic of Macedonia |
| <input type="checkbox"/> BG Bulgaria                              | <input type="checkbox"/> MN Mongolia                                  |
| <input type="checkbox"/> BR Brazil                                | <input type="checkbox"/> MW Malawi                                    |
| <input type="checkbox"/> BY Belarus                               | <input type="checkbox"/> MX Mexico                                    |
| <input checked="" type="checkbox"/> CA Canada                     | <input type="checkbox"/> NO Norway                                    |
| <input type="checkbox"/> CH and LI Switzerland and Liechtenstein  | <input type="checkbox"/> NZ New Zealand                               |
| <input type="checkbox"/> CN China                                 | <input type="checkbox"/> PL Poland                                    |
| <input type="checkbox"/> CU Cuba                                  | <input type="checkbox"/> PT Portugal                                  |
| <input type="checkbox"/> CZ Czech Republic                        | <input type="checkbox"/> RO Romania                                   |
| <input type="checkbox"/> DE Germany                               | <input type="checkbox"/> RU Russian Federation                        |
| <input type="checkbox"/> DK Denmark                               | <input type="checkbox"/> SD Sudan                                     |
| <input type="checkbox"/> EE Estonia                               | <input type="checkbox"/> SE Sweden                                    |
| <input type="checkbox"/> ES Spain                                 | <input type="checkbox"/> SG Singapore                                 |
| <input type="checkbox"/> FI Finland                               | <input type="checkbox"/> SI Slovenia                                  |
| <input type="checkbox"/> GB United Kingdom                        | <input type="checkbox"/> SK Slovakia                                  |
| <input type="checkbox"/> GE Georgia                               | <input type="checkbox"/> SL Sierra Leone                              |
| <input type="checkbox"/> GD Grenada                               | <input type="checkbox"/> ZA South Africa                              |
| <input type="checkbox"/> GH Ghana                                 | <input type="checkbox"/> TJ Tajikistan                                |
| <input type="checkbox"/> GM Gambia                                | <input type="checkbox"/> TM Turkmenistan                              |
| <input type="checkbox"/> HR Croatia                               | <input type="checkbox"/> TR Turkey                                    |
| <input type="checkbox"/> HU Hungary                               | <input type="checkbox"/> TT Trinidad and Tobago                       |
| <input type="checkbox"/> ID Indonesia                             | <input type="checkbox"/> UA Ukraine                                   |
| <input type="checkbox"/> IL Israel                                | <input type="checkbox"/> UA Uganda                                    |
| <input type="checkbox"/> IN India                                 | <input type="checkbox"/> AE United Arab Emirates                      |
| <input type="checkbox"/> IS Iceland                               | <input checked="" type="checkbox"/> US United States of America       |
| <input checked="" type="checkbox"/> JP Japan                      | <input type="checkbox"/> UZ Uzbekistan                                |
| <input type="checkbox"/> KE Kenya                                 | <input type="checkbox"/> VN Vietnam                                   |
| <input type="checkbox"/> KG Kyrgyzstan                            | <input type="checkbox"/> YU Yugoslavia                                |
| <input type="checkbox"/> KP Democratic People's Republic of Korea | <input type="checkbox"/> ZW Zimbabwe                                  |
| <input type="checkbox"/> KR Republic of Korea                     |   |
| <input type="checkbox"/> KZ Kazakhstan                            |   |
| <input type="checkbox"/> LC Saint Lucia                           |   |
| <input type="checkbox"/> LK Sri Lanka                             |   |
| <input type="checkbox"/> LR Liberia                               |   |

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet.

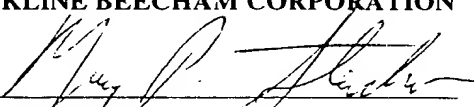
- ☐ .....
- ☐ .....
- ☐ .....

In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of none. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)

**Supplemental Box** *If the Supplemental Box is not used, this sheet need not be included in the request**Use this box in the following cases:***1.** *If, in any of the Boxes, the space is insufficient to furnish all the information:**in particular*(i) *if more than three persons are involved as applicants and/or inventors and no "continuation sheet" is available:**in such cases, write "Continuation of Box No...." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the box in which the space was insufficient;*(ii) *if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked:**in such case, write "Continuation of Box III" and indicate for each additional person the same type of information as required in Box No. III;*(iii) *if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America:**in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State or States (and/or, where applicable, European or OAPI patent) for the purposes of which the named person is applicant;*(iv) *if, in addition to the agent(s) indicated in Box No. IV, there are further agents:**in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name the State or States (and/or, where applicable, European or OAPI patent) for the purposes of which the named person is inventor;*(v) *if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," "certificate of addition," or "inventor's certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "Continuation" or "Continuation-in-part":**in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*(vi) *if there are more than three earlier applications whose priority is claimed:**in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;***2.** *If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty:**in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.**in such case, write "Statement Concerning Non-Prejudicial Disclosures or Exceptions to Lack of Novelty" and furnish that statement below.***Continuation of Box No. IV:**

Baumeister, Kirk  
 Dinner, Dara L.  
 Dustman, Wayne J.  
 Geiger, Kathleen W.  
 Gimmi, Edward R.  
 Hall, Linda E.  
 Han, William T.  
 Hecht, Elizabeth  
 Kanagy, James M.  
 Kerekes, Zoltan  
 King, William T.  
 Kinzig, Charles M.  
 McCarthy, Mary E.  
 Simon, Soma G.  
 Stein-Fernandez, Nora  
 Venetianer, Stephen  
 Williams, Janice E.

**All of SmithKline Beecham Corporation, address as indicated in Box No. IV.**

<b>Box No. VI PRIORITY CLAIM</b>				Further priority claims are indicated in the Supplemental Box <input type="checkbox"/>	
The priority of the following earlier application(s) is hereby claimed:					
Country (in which, or for which, the application was filed)	Filing Date (day/month/year)	Application No.	Office of filing (only for regional or international application)		
item (1) <b>United States of America</b>	<b>(07.07.98)</b> <b>07 July 1998</b>	<b>60/091,944</b>			
item (2)					
item (3)					
Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):					
<input checked="" type="checkbox"/> The receiving Office is hereby requested to transmit to the International Bureau a certified copy of the earlier application(s) identified above at item(s): <b>(1)</b>					
<b>Box No. VII EARLIER SEARCH</b>					
Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:					
Country(or regional Office)		Date (day/month/year):		Number:	
<b>Box No. VIII CHECK LIST</b>					
This international application contains the following number of sheets:			This international application is accompanied by the item(s) marked below:		
1. request	: 05	sheets	1. <input checked="" type="checkbox"/> separate signed power of attorney	5. <input checked="" type="checkbox"/> fee calculation sheet	
2. description	: 12	sheets	2. <input type="checkbox"/> copy of general power of attorney	6. <input type="checkbox"/> separate indications concerning deposited microorganisms.	
3. claims	: 04	sheets	3. <input type="checkbox"/> statement explaining lack of signature	7. <input type="checkbox"/> nucleotide and/or amino acid sequence listing	
4. abstract	: 01	sheets	4. <input type="checkbox"/> priority document(s)	8. <input checked="" type="checkbox"/> other (specify):	
5. figures	: 00	sheets	identified in Box No. VI as item(s):		
<b>Total</b>	<b>: 22</b>	<b>sheets</b>	<b>Transmittal Letter Board Authorization</b>		
Figure No. _____ of the drawings (if any) should accompany the abstract when it is published					
<b>Box No. IX SIGNATURE OF APPLICANT OR AGENT</b>					
Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such is not obvious from reading the request).					
<b>SMITHKLINE BEECHAM CORPORATION</b>					
By: 					
<b>Yuriy P. Stepcho</b> Attorney for the Applicant					
<b>For receiving Office use only</b>					
1. Date of actual receipt of the international application	<b>516 Rec'd PCT/PTO</b>		<b>07 JUL 1999</b>	<b>(07.07.99)</b>	
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:				<input type="checkbox"/> received:	
4. Date of timely receipt of the required corrections under PCT Article 11(2)				<input type="checkbox"/> not received:	
5. International Searching Authority specified by the applicant: <b>ISA/ US</b>			6. <input type="checkbox"/> Transmittal of search copy delayed until search fee is paid		
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Date: 20 May 1999

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## Terbium Chelate for Use as a Label in Fluorescent Immunoassays

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Human serum albumin has been labelled with a terbium complex by means of a reagent prepared from the bis-cyclic anhydride of diethylenetriamine pentaacetic acid and *p*-aminosalicylic acid. This reagent combines high fluorescent intensity with stability at high dilution ( $10^{-9}$  M). The fluorescent conjugate so produced has been used in a simple fluoroimmunoassay, using human serum albumin as a model analyte.

**Keywords:** Fluoroimmunoassay; terbium label; human serum albumin

The use of chelates of rare-earth elements as labels in fluorescent immunoassay<sup>1-4</sup> offers a great improvement in signal to noise ratio over previously used fluorophores. A difficulty with these labels is that those chelate complexes which are stable at high dilution are poorly fluorescent, and those which are highly fluorescent are largely dissociated at high dilution: consequently, separate reagents have been used for attachment and for fluorescent enhancement, an arrangement that hampers the development of homogeneous assays.

The terbium complex of a reagent formed from diethylenetriaminepentaacetic acid anhydride (DTPAA) and *p*-aminosalicylic acid (pAS) is strongly fluorescent, and stable in highly dilute solution ( $10^{-9}$  M). We describe the preparation of this reagent, and illustrate its use in a simple fluoroimmunoassay for human serum albumin.

### Experimental

DTPAA and pAS, sodium salt, were obtained from Sigma (Poole, Dorset). DTPAA is the bis-cyclic anhydride of diethylenetriaminepentaacetic acid (DTPA).<sup>5</sup> DTPAA (0.1 mmol, 36 mg) and triethylamine (0.1 mmol, 14  $\mu$ l) were dissolved in 0.5 ml of dimethyl sulphoxide (DMSO). Sodium *p*-aminosalicylate was dried at 110°C until no further decrease in mass occurred, and 0.1 mmol (18 mg) of the anhydrous salt was dissolved in 0.5 ml of DMSO. The pAS solution was added dropwise to the stirred DTPAA solution and the mixture was stirred for 30 min at room temperature. Human serum albumin was labelled with the DTPA - pAS derivative by adding 0.1 ml of the rapidly stirred suspension to a solution of 50 mg of albumin in 5 ml of 0.1 M phosphate buffer (pH 7) with continuous mixing. The mixture was kept overnight at 4°C and the excess of label was removed by dialysis for 36 h against three 1-l volumes of 9 g l<sup>-1</sup> sodium chloride solution.

For use in a fluoroimmunoassay, 0.4 ml of the labelled albumin preparation was mixed with 0.1 ml of 0.05 M terbium chloride solution and this mixture was diluted 1 + 99 with 0.1 M phosphate buffer (pH 7). The excess of terbium was precipitated as phosphate and could be removed by centrifuging briefly. Diluted label (0.1 ml) was mixed with 0.1 ml of albumin standard solution or diluted plasma [1 + 199 in 0.1 M phosphate buffer (pH 7)] and 0.1 ml of a 1 + 49 dilution of anti-albumin antiserum. After 30 min at room temperature, 1 ml of polyethylene glycol 6000 [200 g l<sup>-1</sup> in 0.1 M phosphate buffer (pH 7)] was added to each tube and the precipitates were sedimented by centrifugation at 1500 g for 20 min. The supernatants were removed by aspiration and the precipitates resuspended in 1.4 ml of 0.1 M phosphate buffer (pH 7). The assay tubes were placed directly in the cell compartment of a Perkin-Elmer MPF-3L spectrofluorimeter. By scanning the 545-nm emission peak, it was possible to correct each reading for background fluorescence.

### Results

The reaction between DTPAA and pAS in DMSO produced a suspension of material that was readily soluble in water. A solution containing equimolar amounts of the product and terbium chloride showed a bright green fluorescence under ultraviolet light, and proved to have an excitation maximum at 312 nm and sharp emission maxima at 488, 545 and 593 nm (uncorrected). The major peaks are those at 488 and 545 nm and, of these, the 545-nm peak is better resolved from the background fluorescence (Fig. 1). On dilution with water, no decrease in fluorescence was apparent, and as little as  $10^{-12}$  mol of the chelate could be detected in a 2-ml sample volume. The DTPA - pAS terbium complex was also fully fluorescent in 0.1 M phosphate buffer. This is in marked contrast to the  $\beta$ -diketone chelates of europium, which rapidly lose their fluorescence at concentrations below about  $10^{-6}$  M, and are efficiently quenched by phosphate ion. The fluorescence intensity showed only a small variation with pH in the range 4-11, with a broad maximum at pH 8-9.

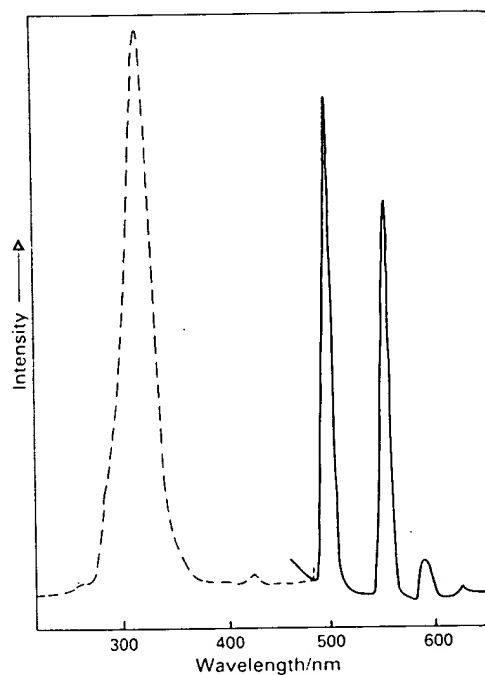


Fig. 1. Excitation (broken line) and emission (solid line) spectra of albumin labelled with the terbium chelate of DTPA - pAS

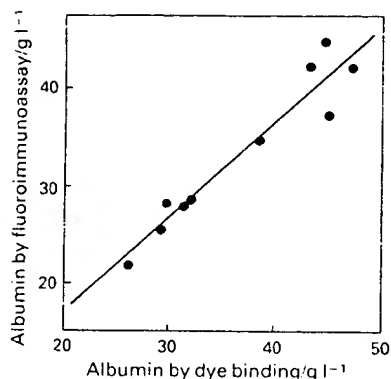


Fig. 2. Comparison of albumin concentrations measured by fluoroimmunoassay and by a dye-binding method

Gel chromatography (Sephadex G-75; Pharmacia, Hounslow, Middlesex) of the albumin-chelate mixture, to which terbium had been added, showed two peaks of 545-nm fluorescent material. One of these peaks eluted with the void volume of the column and was not removed by dialysis. Terbium-labelled albumin could be diluted without a decrease in fluorescence, and as little as 250 ng (5 pmol) could be detected. Terbium fluorescence in the absence of the coupling reagent was negligible. No change in fluorescence occurred when the labelled albumin was bound to anti-albumin antibodies. As shown in Fig. 2, fluoroimmunoassay of

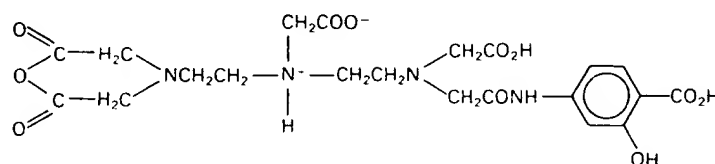


Fig. 3. Presumed structure of the product of reaction between 1 mol of DTPAA and 1 mol of pAS

albumin showed good correlation with a dye-binding method using a parallel multi-channel analyser (Monitor International, Storrington, Sussex) ( $n = 10$ ,  $r = 0.96$ , slope = 0.94, intercept  $-1.8 \text{ g l}^{-1}$ ) and a coefficient of variation for 20 replicate samples of 4.9% at  $150 \text{ mg l}^{-1}$  (equivalent to  $30 \text{ g l}^{-1}$  before dilution).

Time-resolved measurements were made on a Perkin-Elmer LS-5 fluorescence spectrometer. The decay (545 nm) was exponential with lifetimes of 1.58 ms for the unconjugated chelate and 1.52 ms for the chelate bound to albumin.

### Discussion

Several approaches to the use of rare-earth elements as labels in immunoassays have been adopted. Diketonate complexes have been attached to protein by the use of an isothiocyanate derivative of a diketone<sup>1</sup> or by the use of a mixed phenanthroline-diketonate complex carrying an isothiocyanate group on the phenanthroline.<sup>6</sup> These complexes are particularly strongly fluorescent, but dissociate so readily in dilute solution that they are of little practical use as labels. The dissociation of the simple 3:1 diketonate complexes can be suppressed by the use of excess of reagent, but this approach is not feasible for a chelate of this type linked to protein via one of the ligand molecules.

In the assays described by workers at Wallace Laboratories,<sup>2-4</sup>  $\beta$ -diketonates are used to enhance europium fluorescence, but the europium is initially attached to a label using a bifunctional derivative of ethylenediaminetetraacetic acid (EDTA).<sup>7,8</sup> After immunoreaction, the lanthanide is dissociated from the label and the diketonate is formed in a solution containing an excess of reagent. The use of bifunctional derivatives of EDTA has been described in a simpler system, in which the fluorescence of the terbium complex of phenyl-

EDTA attached to protein was measured directly. The quantum yield of this complex is low, and adequate sensitivity was achieved by the use of laser excitation and time-resolved detection.

The transferrin complex of terbium has been linked to gentamicin with a view to using it in fluorescent immunoassay,<sup>10</sup> but the detection sensitivity was relatively low.

The reagent described here is presumed to have the structure shown in Fig. 3. It combines the advantages of two classes of lanthanide chelate: the polyaminopolycarboxylate portion of the molecule ensures that the metal is tightly bound, whilst the aromatic moiety provides an absorbing centre. The aromatic ring carries chelating groups that may enhance both the stability of the complex and the efficiency of energy transfer to the rare-earth ion. The synthesis of the reagent is simple, and the same procedure can be used to prepare a family of similar compounds. Work on such compounds, and their lanthanide complexes, is continuing.

The availability of a lanthanide label that is highly fluorescent in its own right opens the way for the development of highly sensitive automated fluoroimmunoassays. In this study we have corrected the fluorescence readings by taking advantage of the narrow band width of the lanthanide emission; however, with suitable instrumentation, it will be possible to make time-resolved measurements. The long fluorescence lifetime of the complex means that background fluorescence can be eliminated and the sensitivity increased by several orders of magnitude.

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